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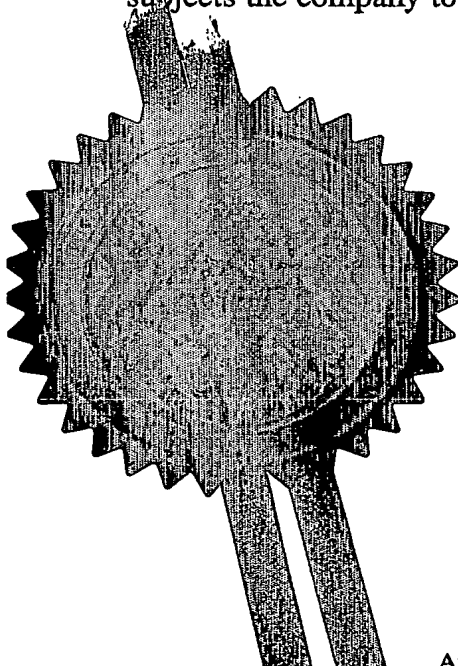
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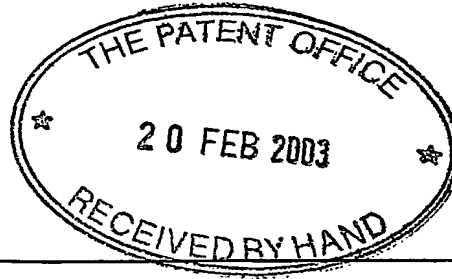


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Patent 1977  
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# Request for grant of a patent

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1.	Your reference	CDM/DCM/61867/000		
2.	Patent application number (The Patent Office will fill in this part)	0303924.5		20 FEB 2003
3.	Full name, address and postcode of the or of each applicant (underline all surnames)	SOLEXA LIMITED Chesterford Research Park Little Chesterford Nr. Saffron Walden Essex CB10 1XL  Patents ADP number (if you know it) 7533698002 If the applicant is a corporate body, give the country/state of its incorporation GB		
4.	Title of the invention	Modified Nucleotides		
5.	Name of your agent (if you have one)	BOULT WADE TENNANT		
	"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)	VERULAM GARDENS 70 GRAY'S INN ROAD LONDON WC1X 8BT  Patents ADP number (if you know it) 42001		
6.	If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number	Country	Priority application number (if you know it)	Date of filing (day/month/year)
7.	If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application		Date of filing (day / month / year)
8.	Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if: a) any applicant named in part 3 is not an inventor, or b) there is an inventor who is not named as an applicant, or c) any named applicant is a corporate body. See note (d))	Yes		

# Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form

Description 32

Claim(s) 6

Abstract

Drawing(s) 3 + 5 *pk*

10. If you are also filing any of the following, state how many against each item.

Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (*Patents Form 7/77*)

Request for preliminary examination and search (*Patents Form 9/77*)

Request for substantive examination (*Patents Form 10/77*)

Any other documents  
(Please specify)

11

I/We request the grant of a patent on the basis of this application.

Signature

Date

*Bonk Wade Temant*

20 February 2003

12. Name and daytime telephone number of person to contact in the United Kingdom

Colm Murphy  
020 7430 7500

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## Notes

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### Modified Nucleotides

The invention relates to modified nucleotides. In particular, this invention discloses nucleotides  
5 having a removable protecting group, their use in polynucleotide sequencing methods and a method for chemical deprotection of the protecting group.

Advances in the study of molecules have been led,  
10 in part, by improvement in technologies used to characterise the molecules or their biological reactions. In particular, the study of the nucleic acids DNA and RNA has benefited from developing technologies used for sequence analysis and the study  
15 of hybridisation events.

An example of the technologies that have improved the study of nucleic acids is the development of fabricated arrays of immobilised nucleic acids. These  
20 arrays consist typically of a high-density matrix of polynucleotides immobilised onto a solid support material. See, e.g., Fodor et al., *Trends Biotech.* 12:19-26, 1994, which describes ways of assembling the nucleic acids using a chemically sensitized glass  
25 surface protected by a mask, but exposed at defined areas to allow attachment of suitably modified nucleotide phosphoramidites. Fabricated arrays can also be manufactured by the technique of "spotting" known polynucleotides onto a solid support at  
30 predetermined positions (e.g., Stimpson et al., *Proc. Natl. Acad. Sci. USA* 92:6379-6383, 1995).

Sequencing by synthesis of DNA ideally requires the controlled (i.e. one at a time) incorporation of  
35 the correct complementary nucleotide opposite the oligonucleotide being sequenced. This allows for

accurate sequencing by adding nucleotides in multiple cycles as each nucleotide residue is sequenced one at a time, thus preventing an uncontrolled series of incorporations occurring. The incorporated nucleotide is read using an appropriate label attached thereto before removal of the label moiety and the subsequent next round of sequencing. In order to ensure only a single incorporation occurs, a reversible structural modification ("blocking group") of the sequencing nucleotides is required to ensure a single nucleotide incorporation but which then prevents any further nucleotide incorporation into the polynucleotide chain. The blocking group must then be removable, under reaction conditions which do not interfere with the integrity of the DNA being sequenced. The sequencing cycle can then continue with the incorporation of the next blocked, labelled nucleotide. In order to be of practical use, the entire process should consist of high yielding, highly specific chemical and enzymatic steps to facilitate multiple cycles of sequencing.

To be useful in DNA sequencing, nucleotide, and more usually nucleotide triphosphates, generally require a 3'OH-blocking group so as to prevent the polymerase used to incorporate it into a polynucleotide chain from continuing to replicate once the base on the nucleotide is added. There are many limitations on the suitability of a molecule as a blocking group. It must be such that it prevents additional nucleotide molecules from being added to the polynucleotide chain whilst simultaneously being easily removable from the sugar moiety without causing damage to the polynucleotide chain. Furthermore, the modified nucleotide must be tolerated by the polymerase or other appropriate enzyme used to incorporate it into the polynucleotide chain. The

ideal blocking group will therefore exhibit long term stability, be efficiently incorporated by the polymerase enzyme, cause total blocking of secondary or further incorporation and have the ability to be removed under mild conditions that do not cause damage to the polynucleotide structure, preferably under aqueous conditions. These stringent requirements are formidable obstacles to the design and synthesis of the requisite modified nucleotides.

Reversible blocking groups for this purpose have been described previously but none of them generally meet the above criteria for polynucleotide, e.g. DNA-compatible chemistry.

Metzker et al., (*Nucleic Acids Research*, 22(20): 4259-4267, 1994) discloses the synthesis and use of eight 3'-modified 2-deoxyribonucleoside 5'-triphosphates (3'-modified dNTPs) and testing in two DNA template assays for incorporation activity. The 3'-modified dNTPs included 3'-allyl deoxyriboadenosine 5'-triphosphate (3'-allyl dATP). However, the 3'-allyl blocked compound was not used to demonstrate a complete cycle of termination, deprotection and reinitiation of DNA synthesis: the only test results presented were those which showed the ability of this compound to terminate DNA synthesis in a single termination assay, out of eight such assays conducted, each conducted with a different DNA polymerase.

WO02/29003 (The Trustees of Columbia University in the City of New York) describes a sequencing method which may include the use of an allyl protecting group to cap the 3'-OH group on a growing strand of DNA in a polymerase reaction. The allyl group is introduced according to the procedure of Metzker (*infra*) and is said to be removed by using methodology reported by

Kamal et al (*Tet. Let.*, 40, 371-372, 1999).

5       The Kamal deprotection methodology employs sodium  
iodide and chlorotrimethylsilane so as to generate *in*  
*situ* iodotrimethylsilane, in acetonitrile solvent,  
quenching with sodium thiosulfate. After extraction  
into ethyl acetate and drying (sodium sulfate), then  
concentration under reduced pressure and column  
10       chromatography (ethyl acetate:hexane; 2:3 as eluant),  
free alcohols were obtained in 90-98% yield.

15       In WO02/29003, the Kamal allyl deprotection is  
suggested as being directly applicable in DNA  
sequencing without modification, the Kamal conditions  
being mild and specific.

20       While Metzker reports on the preparation of a  
3'-allyl-blocked nucleotide or nucleotide and  
WO02/29003 suggests the use of the allyl functionality  
as a 3'-OH cap during sequencing, neither of these  
documents actually teaches the deprotection of 3'-  
allylated hydroxyl group in the context of a  
sequencing protocol. Whilst the use of an allyl group  
as a hydroxyl protecting group is well known - it is  
25       easy to introduce and is stable across the whole pH  
range and to elevated temperatures - there is to date,  
no concrete embodiment of the successful cleavage of a  
3'-allyl group under DNA compatible conditions, i.e.  
conditions under which the integrity of the DNA is not  
30       wholly or partially destroyed. In other words, it has  
not been possible hitherto to conduct DNA sequencing  
using 3'-OH allyl-blocked nucleotides.

35       The Kamal methodology is inappropriate to conduct  
in aqueous media since the TMS chloride will hydrolyse  
preventing the *in situ* generation of TMS iodide.



Attempts to carry out the Kamal deprotection (in acetonitrile) in sequencing have proven unsuccessful in our hands.

5           The present invention is based on the surprising development of a completely new method of allyl deprotection. Our procedure is of broad applicability to the deprotection of virtually all allyl-protected hydroxyl functionality and may be effected in aqueous  
10 solution, in contrast to the methodology of Kamal et al. (which is effected in acetonitrile) and to the other methods known generally in the prior art which are highly oxygen-and moisture-sensitive. The methodology makes use of a water-soluble transition  
15 metal catalyst formed from a transition metal and at least partially water-soluble ligands. In aqueous solution these form at least partially water-soluble transition metal complexes. By aqueous solution herein is meant a liquid comprising at least 20 vol%,  
20 preferably at least 50%, for example at least 75 vol%, particularly at least 95 vol% and especially greater than above 98 vol%, ideally 100 vol% of water as the continuous phase.

25           As those skilled in the art will appreciate, the allyl group may be used to protect not only the hydroxyl group but also thiol and amine functionalities. Moreover allylic esters may be formed from the reaction between carboxylic acids and  
30 allyl halides, for example. Primary or secondary amides may also be protected using methods known in the art. The novel deprotection methodology described herein may be used in the deprotection of all these allylated compounds, e.g. allyl esters and mono- or  
35 bisallylated primary amines or allylated amides, or in the deprotection of allylated secondary amines. The method is also suitable in the deprotection of allyl

esters and thioethers.

Viewed from a first aspect, therefore, the invention provides a method of converting a compound of formula R-O-allyl, R<sub>2</sub>N(allyl), RNH(allyl), RN(allyl)<sub>2</sub> or R-S-allyl to a corresponding compound in which the allyl group is removed and replaced by hydrogen, said method comprising the steps of reacting a compound of formula R-O-allyl, R<sub>2</sub>N(allyl), RNH(allyl), RN(allyl)<sub>2</sub> or R-S-allyl in aqueous solution with a transition metal comprising a transition metal and one or more ligands selected from the group comprising water-soluble phosphine and water-soluble nitrogen-containing phosphine ligands, wherein the or each R is a water-soluble biological molecule.

Viewed from another aspect, the invention provides a 3'-O-allyl nucleotide or nucleoside which nucleotide or nucleoside comprises a detectable label linked to the base of the nucleoside or nucleotide by a cleavable linker.

In a further aspect, the invention provides a polynucleotide comprising a 3'-O-allyl nucleotide or nucleoside which nucleotide or nucleoside comprises a detectable label linked to the base of the nucleoside or nucleotide by a cleavable linker.

In an even further aspect the invention provides a method of controlling the incorporation of a nucleotide molecule complementary to the nucleotide in a target single-stranded polynucleotide in a synthesis or sequencing reaction comprising incorporating into the growing complementary polynucleotide 3'-O-allyl nucleotide, the incorporation of said molecule preventing or blocking introduction of subsequent

nucleoside or nucleotide molecules into  
said growing complementary polynucleotide.

5 Viewed from a further aspect still, the invention  
provides a method for determining the sequence of a  
target single-stranded polynucleotide, comprising  
monitoring the sequential incorporation of  
complementary nucleotides, wherein at least one  
10 incorporation of the nucleotide is a 3'-O-allyl  
nucleotide which comprises a detectable label linked  
to the base of the nucleoside or nucleotide by a  
cleavable linker and wherein the identity of the 3'-O-  
allyl nucleotide incorporated is determined by  
15 detecting the label linked to the base, said blocking  
group and said label being removed prior to  
introduction of the next complementary nucleotide.

In an even further aspect, the invention provides  
a method for determining the sequence of a target  
20 single-stranded polynucleotide, comprising:

(a) providing a plurality of different 3'-O-  
allyl protected nucleotides linked from the base to a  
detectable label by a cleavable linker and wherein the  
detectable label linked to each type of nucleotide can  
25 be distinguished upon detection from the detectable  
label used for other types of nucleotides;

(b) incorporating the nucleotide into the  
complement of the target single-stranded  
polynucleotide;

30 (c) detecting the label of the nucleotide of  
(b), thereby determining the type of nucleotide  
incorporated;

(d) removing the label of the nucleotide of (b)  
and the allyl blocking group; and

35 (e) optionally repeating steps (b)-(d) one or  
more times;

thereby determining the sequence of a target

single-stranded polynucleotide.

Additionally, in another aspect, the invention provides a kit, comprising:

- 5 (a) a plurality of different 3'O-allyl individual nucleotides; and
- (b) packaging materials therefor.

10 The nucleosides or nucleotides of the present invention comprise a purine or pyrimidine base and a ribose or deoxyribose sugar moiety which has an allyl group covalently attached thereto, preferably at the 3'O position, which renders the molecules useful in techniques requiring blocking of the 3'-OH group to  
15 prevent incorporation of additional nucleotides, such as for example in sequencing reactions, polynucleotide synthesis, nucleic acid amplification, nucleic acid hybridisation assays, single nucleotide polymorphism studies, and other such techniques.

20 Once the allyl group has been removed, it is possible to incorporate another nucleotide to the free 3'-OH group.

25 The molecule can be linked via the base to a detectable label by a desirable linker, which label may be a fluorophore, for example. The linker can be acid labile, photolabile or contain a disulfide linkage.

30 Preferred labels and linkages included those disclosed in our co-pending International patent application number PCT/GB02/005474 filed on 4 December 2002 and entitled "Labelled Nucleotides" and in United  
35 States Patent Application Serial 10/227,131 filed 23 August 2002.

In the method of controlling the incorporation of a nucleotide molecule complementary to the nucleotide in a target single stranded polynucleotide in a synthesis or sequencing reaction of the invention, the  
5 incorporation of the molecule may be accomplished via a terminal transferase, a polymerase or a reverse transcriptase.

10 In the methods for determining the sequence of a target single stranded polynucleotide comprising monitoring the sequential incorporation of complementary nucleotides of the invention, it is preferred that the blocking group and the label may be removed in a single chemical treatment step. Thus, in  
15 a preferred embodiment of the invention, the blocking group is cleaved simultaneously with the label. Furthermore, preferably the blocked and labelled modified nucleotide constructs of the nucleotide bases A, T, C and G are recognised as substrates by the same  
20 polymerase enzyme.

In the methods described herein, each of the nucleotides can be brought into contact with the target sequentially, with removal of non-incorporated  
25 nucleotides prior to addition of the next nucleotide, where detection and removal of the label and the blocking group is carried out either after addition of each nucleotide, or after addition of all four nucleotides.

30 In the methods, all of the nucleotides can be brought into contact with the target simultaneously, i.e., a composition comprising all of the different nucleotides is brought into contact with the target,  
35 and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label and the blocking group.

The methods can comprise a first step and a second step, where in the first step, a first composition comprising two of the four types of modified nucleotides is brought into contact with the target, and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label and the allyl blocking group, and where in the second step, a second composition comprising the two nucleotides not included in the first composition is brought into contact with the target, and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label and blocking group, and where the first steps and the second step can be optionally repeated one or more times.

The methods described herein can also comprise a first step and a second step, where in the first step, a composition comprising one of the four nucleotides is brought into contact with the target, and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label and allyl blocking group, and where in the second step, a second composition comprising the three nucleotides not included in the first composition is brought into contact with the target, and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label and allyl blocking group, and where the first steps and the second step can be optionally repeated one or more times.

The methods described herein can also comprise a first step and a second step, where in the first step, a first composition comprising three of the four nucleotides is brought into contact with the target, and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label and

allyl blocking group and where in the second step, a composition comprising the nucleotide not included in the first composition is brought into contact with the target, and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label and allyl blocking group, and where the first steps and the second step can be optionally repeated one or more times.

10       The kits of the invention include: (a) individual nucleotides according to the hereinbefore described invention, where each nucleotide has a base that is linked to a detectable label via a cleavable linker, and where the detectable label linked to each  
15       nucleotide can be distinguished upon detection from the detectable label used for other three nucleotides; and (b) packaging materials therefor. The kit can further include an enzyme for incorporating the nucleotide into the complementary nucleotide chain and  
20       buffers appropriate for the action of the enzyme in addition to appropriate chemicals for removal of the allyl blocking group and the detectable label, which can preferably be removed by the same chemical treatment step.

25       The nucleotides/nucleosides are suitable for use in many different DNA-based methodologies, including DNA synthesis and DNA sequencing protocols.

30       The invention may be understood with reference to the attached drawings in which:

      Fig. 1 shows exemplary nucleotide structures useful in the invention. For each structure, X can be H, phosphate, diphosphate or triphosphate.  $R_1$  and  $R_2$   
35       can be the same or different, and can be selected from H, OH, or any group which can be transformed into an

OH, including, but not limited to, a carbonyl. Some suitable functional groups for  $R_1$  and  $R_2$  include the structures shown in Fig. 4.

Fig. 2 shows structures of linkers useful in certain aspects of the invention, including (1) disulfide linkers and acid labile linkers, (2) dialkoxybenzyl linkers, (3) Sieber linkers, (4) indole linkers and (5) *t*-butyl Sieber linkers.

Fig. 3 shows some functional molecules useful in the invention, including some cleavable linkers and some suitable hydroxyl protecting groups. In these structures,  $R_1$  and  $R_2$  may be the same or different, and can be H, OH, or any group which can be transformed into an OH group, including a carbonyl.  $R_3$  represents one or more substituents independently selected from alkyl, alkoxyl, amino or halogen groups. Alternatively, cleavable linkers may be constructed from any labile functionality used on the allyl 3'-OH block.  $R_4$  and  $R_5$  can be H or alkyl, and  $R_6$  can be alkyl, cycloalkyl, alkenyl, cycloalkenyl or benzyl. X can be H, phosphate, diphosphate or triphosphate.

The present invention relates to nucleotide or nucleoside molecules that are modified by the reversible covalent attachment of a 3'-OH allyl blocking group thereto, and which molecule may be used in reactions where blocked nucleotide or nucleoside molecules are required, such as in sequencing reactions, polynucleotide synthesis and the like.

The allyl group may be introduced into the 3'-position using standard literature procedures such as that used by Metzker (*infra*).



The allyl groups are removed by reacting in aqueous solution a compound of formula  $R-O-allyl$ ,  $R_2N(allyl)$ ,  $RNH(allyl)$ ,  $RN(allyl)_2$  or  $R-S-allyl$  (wherein R is a water-soluble biological molecule) with a transition metal, wherein said transition metal is capable of forming a metal allyl complex, in the presence of one or more ligands selected from the group comprising water-soluble phosphine and water-soluble mixed nitrogen-phosphine ligands.

The water-soluble biological molecule is not particularly restricted provided, of course, it contains one or more hydroxyl, acid, amino, amide or thiol functionalities protected with an allyl group. Allyl esters are examples of compounds of formula  $R-O-allyl$ . Preferred functionalities are hydroxyl and amino.

As used herein the term biological molecule is used to embrace any molecules or class of molecule which performs a biological role. Such molecules include for example, polynucleotides such as DNA and RNA, oligonucleotides and single nucleotides. In addition, peptides and peptide mimetics, such as enzymes and hormones etc., are embraced by the invention. Compounds which comprise a secondary amide linkage, such as peptides, or a secondary amine, where such compounds are allylated on the nitrogen atom of the secondary amine or amide, are examples of compounds of formula  $R_2N(allyl)$  in which both R groups belong to the same biological molecule. Particularly preferred compounds however are polynucleotides, (including oligonucleotides) and nucleotides and nucleosides, preferably those which contain one base

to which is attached a detectable label linked through a cleavable linker. Such compounds are useful in the determination of sequences of oligonucleotides as described herein.

5

Transition metals of use in the invention are any which may form metal allyl complexes, for example platinum, palladium, rhodium, ruthenium, osmium and iridium. Palladium is preferred.

10

The transition metal, e.g. palladium, is conveniently introduced as a salt, e.g. as a halide. Mixed salts such as  $\text{Na}_2\text{PdCl}_4$  may also be used. Other appropriate salts and compounds will be readily determined by the skilled man and are commercially available, e.g. from Aldrich Chemical Company.

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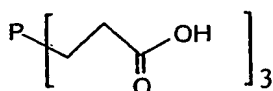
Suitable ligands are any phosphine or mixed nitrogen-phosphine ligands known to those skilled in the art, characterised in that the ligands are derivatised so as to render them water-soluble, e.g. by introducing one or more sulfonate, amine, hydroxyl (preferably a plurality of hydroxyl) or carboxylate residues. Where amine residues are present, formation of amine salts may assist the solubilisation of the ligand and thus the metal-allyl complex. Examples of appropriate ligands are triaryl phosphines, e.g. triphenyl phosphine, derivatised so as to make them water soluble. Sulfonate-containing ligands are particularly preferred, an example of such being the trisodium salt of 3,3',3''-phosphinidynetris (benzenesulfonic acid) which is commercially available from Aldrich Chemical Company.

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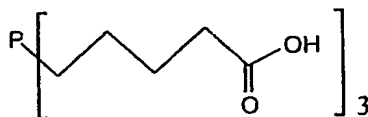
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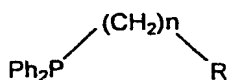
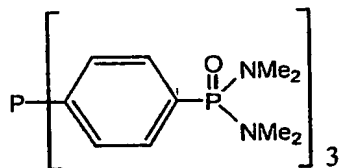
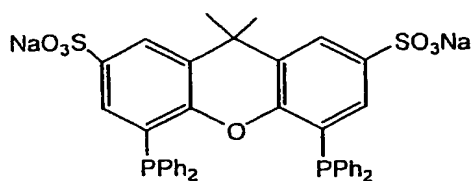
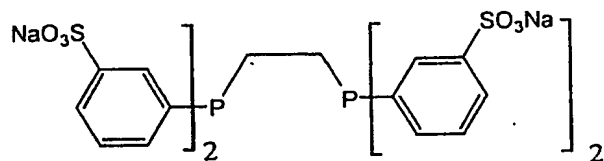
Other ligands which may be used to include the following:



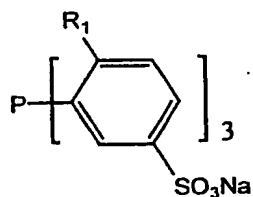
as the hydrochloride salt, neutral compound and the trisodium salt



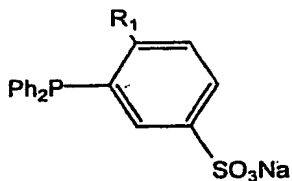
as the hydrochloride salt, neutral compound and the trisodium salt



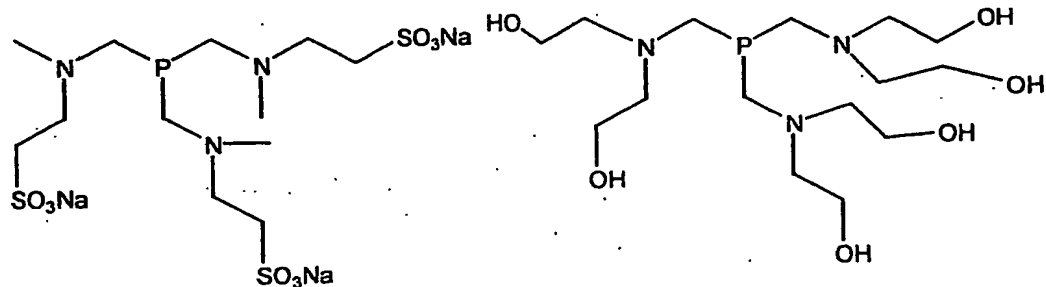
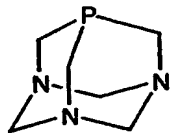
$n=1-5$   
 $\text{R}=\text{PO}_3\text{Na}_2,$   
 $\text{CO}_2\text{Na},$   
 $\text{SO}_3\text{Na},$   
 $\text{OH}$



$\text{R}_1=\text{OMe}, \text{CO}_2\text{H}, \text{CO}_2\text{Na}$



$\text{R}_1=\text{OMe}, \text{CO}_2\text{H}, \text{CO}_2\text{Na}$



The skilled man will be aware that the atoms chelated to the transition metal in the water soluble complex may be part of mono- or polydentate ligands. Some such polydentate ligands are shown above. Whilst  
5 monodentate ligands are preferred, the invention thus also embraces methods which use water-soluble bi-, tri-, tetra-, penta- and hexadentate water-soluble phosphine and water-soluble nitrogen-containing phosphine ligands

10

The various aspects of the invention are of particular utility in sequencing polynucleotides wherein the 3'-OH is allylated. However, when present, the 2'-OH is equally amenable to allylation,  
15 and to deprotection according to the method of the invention if necessary. In fact any allylated alcohol may be deprotected according to the method of the invention. Preferred allylated alcohols, however, are those derived from primary and secondary alcohols.  
20 Particularly preferred are allylated nucleosides and nucleotides as described herein. It is possible to deprotect tertiary allylated alcohols - the reaction is simply slower (although deprotection may be in such, and other deprotections of this invention, accelerated if necessary by heating the solution, e.g.  
25 to 40 °C, preferably 50 °C or higher such as approximately 60 °C or even up to 80 °C).

30

It is also possible to deprotect allylated primary or secondary amines and allylated thiols.

As noted earlier, the aqueous solution in which deprotection is effected need not be 100% (as the continuous phase). However, substantially pure water

(e.g. at least 98 vol% preferably about 100 vol%) is preferred. Cosolvents are generally not required although they can assist in the solubilisation of the allylated substrate for the deallylation. Generally, biomolecules are readily soluble in water (e.g. pure water) in which the deprotection reaction described herein may be effected. If desirable, one or more water-miscible cosolvents may be employed.

Appropriate solvents include acetonitrile or dimethylsulfoxide, methanol, ethanol and acetone, methanol being preferred. Less preferred solvents include tetrahydrofuran (THF) and dioxane.

In the method of allyl deprotection according to the invention, a soluble metal complex is formed comprising a transition metal and one or more water-soluble phosphine and water-soluble nitrogen-containing phosphine ligands. More than one type of water-soluble phosphine/nitrogen-containing phosphine ligand may be used in a deallylation reaction although generally only one type of these classes of ligand will be used in a given reaction. We believe the deallylation reaction to be catalytic. Accordingly, the quantity of transition metal, e.g. palladium, may be less than 1 mol% (calculated relative to the allyl-protected compound to be deprotected). Advantageously the amount of catalyst may be much less than 1 mol%, e.g. <0.50 mol%, preferably <0.10 mol%, particularly <0.05mol%. Even lower quantities of metal may be used, for example <0.03 or even <0.01 mol%. As those skilled in the art will be aware, however, as quantity of catalyst is reduced, so too is the speed of the reaction. The skilled man will be able to judge, in any instance, the precise quantity of transition metal

and thus catalyst most optimally suited to any particular deallylation reaction.

5 In contrast to the amount of metal required in forming the active catalyst, the quantity of water-soluble phosphorus-containing ligand(s) used must be greater than 1 molar equivalent (again calculated relative to the allyl-protected compound to be deprotected). Preferably greater than 4, e.g. greater  
10 than 6, for example 8-12 molar equivalents of ligand may be used. Even higher quantities of ligand e.g. >20 mol% may be used if desired.

15 Without wishing to be bound by theory, it is believed that the phosphine/nitrogen-containing phosphine ligand performs two distinct roles in the deallylation reaction. Firstly, it effects solubilisation of the transition metal to form the desired water-soluble catalyst; and secondly it is  
20 oxidised in the reaction forming water-soluble phosphorus (V) species. In this way, the ligand is consumed during the reaction and may be conveniently washed away. Optionally, the phosphine assists in cleavage of the detectable label when the deallylation  
25 reaction is carried out during a sequencing protocol.

The skilled man will be able to determine the quantity of ligand best suited to any individual reaction.

30

As is known in the art, a "nucleotide" consists of a nitrogenous base, a sugar, and one or more phosphate groups. They are monomeric units of a nucleic acid sequence. In RNA, the sugar is a ribose,

and in DNA a deoxyribose, i.e. a sugar lacking a hydroxyl group that is present in ribose. The nitrogenous base is a derivative of purine or pyrimidine. The purines are adenine (A) and guanine (G), and the pyrimidines are cytosine (C) and thymine (T) (or in the context of RNA, uracil (U)). The C-1 atom of deoxyribose is bonded to N-1 of a pyrimidine or N-9 of a purine. A nucleotide is also a phosphate ester or a nucleoside, with esterification occurring on the hydroxyl group attached to C-5 of the sugar. Nucleotides are usually mono, di- or triphosphates.

A "nucleoside" is structurally similar to a nucleotide, but is missing the phosphate moieties. An example of a nucleoside analog would be one in which the label is linked to the base and there is no phosphate group attached to the sugar molecule.

Although the base is usually referred to as a purine or pyrimidine, the skilled person will appreciate that derivatives and analogs are available which do not alter the capability of the nucleotide or nucleoside to undergo Watson-Crick base pairing. "Derivative" or "analog" means a compound or molecule whose core structure is the same as, or closely resembles that of, a parent compound, but which has a chemical or physical modification, such as a different or additional side groups, which allows the derivative nucleotide or nucleoside to be linked to another molecule. For example, the base can be a deazapurine. The derivatives should be capable of undergoing Watson-Crick pairing. "Derivative" and "analog" also mean a synthetic nucleotide or nucleoside derivative having modified base moieties and/or modified sugar

moieties. Such derivatives and analogs are discussed in, e.g., Scheit, Nucleotide Analogs (John Wiley & Son, 1980) and Uhlman et al., Chemical Reviews 90:543-584, 1990. Nucleotide analogs can also  
5 comprise modified phosphodiester linkages, including phosphorothioate, phosphorodithioate, alkylphosphonate, phosphoranilidate and phosphoramidate linkages. The analogs should be capable of undergoing Watson-Crick base pairing.  
10 "Derivative" and "analog", as used herein, may be used interchangeably, and are encompassed by the terms "nucleotide" and "nucleoside" as defined herein.

In the context of the present invention, the term  
15 "incorporating" means becoming part of a nucleic acid (eg DNA) molecule or oligonucleotide or primer. An oligonucleotide refers to a synthetic or natural molecule comprising a covalently linked sequence of nucleotides which are formed by a phosphodiester or  
20 modified phosphodiester bond between the 3' position of the pentose on one nucleotide and the 5' position of the pentose on an adjacent nucleotide.

The term "alkyl" covers straight chain, branched  
25 chain and cycloalkyl groups. Unless the context indicates otherwise, the term "alkyl" refers to groups having 1 to 8 carbon atoms, and typically from 1 to 6 carbon atoms, for example from 1 to 4 carbon atoms. Examples of alkyl groups include methyl, ethyl,  
30 propyl, isopropyl, n-butyl, isobutyl, tert-butyl, n-pentyl, 2-pentyl, 3-pentyl, 2-methyl butyl, 3-methyl butyl, and n-hexyl and its isomers.

Examples of cycloalkyl groups are those having



from 3 to 10 ring atoms, particular examples including those derived from cyclopropane, cyclobutane, cyclopentane, cyclohexane and cycloheptane, bicycloheptane and decalin.

5

The term amino refers to groups of type  $\text{NR}^1\text{R}^2$ , wherein  $\text{R}^1$  and  $\text{R}^2$  are independently selected from hydrogen, a  $\text{C}_{1-6}$  alkyl group (also referred to as  $\text{C}_{1-6}$  alkylamino or di- $\text{C}_{1-6}$  alkylamino).

10

The term "halogen" as used herein includes fluorine, chlorine, bromine and iodine.

15 The nucleotide molecules of the present invention are suitable for use in many different methods where the detection of nucleotides is required.

20 DNA sequencing methods, such as those outlined in U.S. Pat. No. 5,302,509 can be carried out using the nucleotides.

25 The present invention can make use of conventional detectable labels. Detection can be carried out by any suitable method, including fluorescence spectroscopy or by other optical means. The preferred label is a fluorophore, which, after absorption of energy, emits radiation at a defined wavelength. Many suitable fluorescent labels are known. For example, Welch et al. (*Chem. Eur. J.* 5(3):951-960, 1999) discloses dansyl-functionalised fluorescent moieties that can be used in the present invention. Zhu et al. (*Cytometry* 28:206-211, 1997) describes the use of the fluorescent labels Cy3 and Cy5, which can also be used in the present invention.

30

Labels suitable for use are also disclosed in Prober  
et al. (*Science* 238:336-341, 1987); Connell et al.  
(*BioTechniques* 5(4):342-384, 1987), Ansorge et al.  
(*Nucl. Acids Res.* 15(11):4593-4602, 1987) and Smith et  
5 al. (*Nature* 321:674, 1986). Other commercially  
available fluorescent labels include, but are not  
limited to, fluorescein, rhodamine (including TMR,  
texas red and Rox), alexa, bodipy, acridine, coumarin,  
pyrene, benzanthracene and the cyanins.

10 Multiple labels can also be used in the  
invention. For example, bi-fluorophore FRET cassettes  
(*Tet. Let.* 46:8867-8871, 2000) are well known in the  
art and can be utilised in the present invention.  
Multi-fluor dendrimeric systems (*J. Amer. Chem. Soc.*  
15 123:8101-8108, 2001) can also be used.

Although fluorescent labels are preferred, other  
forms of detectable labels will be apparent as useful  
to those of ordinary skill. For example,  
microparticles, including quantum dots (Empodocles et  
20 al., *Nature* 399:126-130, 1999), gold nanoparticles  
(Reichert et al., *Anal. Chem.* 72:6025-6029, 2000) and  
microbeads (Lacoste et al., *Proc. Natl. Acad. Sci USA*  
97(17):9461-9466, 2000) can all be used.

Multi-component labels can also be used in the  
25 invention. A multi-component label is one which is  
dependent on the interaction with a further compound  
for detection. The most common multi-component label  
used in biology is the biotin-streptavidin system.  
Biotin is used as the label attached to the nucleotide  
30 base. Streptavidin is then added separately to enable  
detection to occur. Other multi-component systems are  
available. For example, dinitrophenol has a

commercially available fluorescent antibody that can be used for detection.

5 The invention will be further described with reference to nucleotides. However, unless indicated otherwise, the reference to nucleotides is also intended to be applicable to nucleosides. The invention will also be further described with reference to DNA, although the description will also be applicable to RNA, PNA, and other nucleic acids,  
10 unless otherwise indicated.

The modified nucleotides of the invention use a cleavable linker to attach the label to the nucleotide. The use of a cleavable linker ensures that the label can, if required, be removed after  
15 detection, avoiding any interfering signal with any labelled nucleotide incorporated subsequently.

Cleavable linkers are known in the art, and conventional chemistry can be applied to attach a linker to a nucleotide base and a label. The linker  
20 can be cleaved by any suitable method, including exposure to acids, bases, nucleophiles, electrophiles, radicals, metals, reducing or oxidising agents, light, temperature, enzymes etc. The linker as discussed herein may also be cleaved with the same catalyst used  
25 to cleave the O-allyl bond. Suitable linkers can be adapted from standard chemical blocking groups, as disclosed in Greene & Wuts, Protective Groups in Organic Synthesis, John Wiley & Sons. Further suitable cleavable linkers used in solid-phase  
30 synthesis are disclosed in Guillier et al. (Chem. Rev. 100:2092-2157, 2000).

The use of the term "cleavable linker" is not meant to imply that the whole linker is required to be removed from the nucleotide base. The cleavage site can be located at a position on the linker that  
5 ensures that part of the linker remains attached to the nucleotide base after cleavage.

The linker can be attached at any position on the nucleotide base provided that Watson-Crick base pairing can still be carried out. In the context of  
10 purine bases, it is preferred if the linker is attached via the 7-position of the purine or the preferred deazapurine analogue, via an 8-modified purine, via an N-6 modified adenosine or an N-2 modified guanine. For pyrimidines, attachment is  
15 preferably, via the 5-position on cytosine, thymidine or uracil and the N-4 position on cytosine. Suitable nucleotide structures are shown in Fig. 1. For each structure in Fig. 1 X can be H, phosphate, diphosphate or triphosphate.  $R_1$  and  $R_2$  can be the same or  
20 different, and are selected from H, OH, O-allyl or any other group which can be transformed into an OH, including, but not limited to, a carbonyl, provided that at least one of  $R_1$  and  $R_2$  is O-allyl. Some suitable functional groups for  $R_1$  and  $R_2$  include the  
25 structures shown in Fig. 4.

Suitable linkers are shown in Fig. 3 and include, but are not limited to, disulfide linkers (1), acid labile linkers (2, 3, 4 and 5; including  
dialkoxybenzyl linkers (e.g., 2), Sieber linkers  
30 (e.g., 3), indole linkers (e.g., 4), t-butyl Sieber linkers (e.g., 5)), electrophilically cleavable linkers, nucleophilically cleavable linkers, photocleavable linkers, cleavage under reductive

conditions, oxidative conditions, cleavage via use of safety-catch linkers, and cleavage by elimination mechanisms.

A. Electrophilically cleaved linkers.

5 Electrophilically cleaved linkers are typically cleaved by protons and include cleavages sensitive to acids. Suitable linkers include the modified benzylic systems such as trityl, p-alkoxybenzyl esters and p-alkoxybenzyl amides. Other suitable linkers include  
10 tert-butyloxycarbonyl (Boc) groups and the acetal system.

The use of thiophilic metals, such as nickel, silver or mercury, in the cleavage of thioacetal or other sulfur-containing protecting groups can also be  
15 considered for the preparation of suitable linker molecules.

B. Nucleophilically cleaved linkers.

Nucleophilic cleavage is also a well recognised method in the preparation of linker molecules. Groups  
20 such as esters that are labile in water (i.e., can be cleaved simply at basic pH) and groups that are labile to non-aqueous nucleophiles, can be used. Fluoride ions can be used to cleave silicon-oxygen bonds in groups such as triisopropyl silane (TIPS) or  
25 t-butyldimethyl silane (TBDMS).

C. Photocleavable linkers.

Photocleavable linkers have been used widely in carbohydrate chemistry. It is preferable that the light required to activate cleavage does not affect  
30 the other components of the modified nucleotides. For

example, if a fluorophore is used as the label, it is preferable if this absorbs light of a different wavelength to that required to cleave the linker molecule. Suitable linkers include those based on O-nitrobenzyl compounds and nitroveratryl compounds. 5 Linkers based on benzoin chemistry can also be used (Lee et al., J. Org. Chem. 64:3454-3460, 1999).

D. Cleavage under reductive conditions

There are many linkers known that are susceptible 10 to reductive cleavage. Catalytic hydrogenation using palladium-based catalysts has been used to cleave benzyl and benzyloxycarbonyl groups. Disulfide bond reduction is also known in the art.

E. Cleavage under oxidative conditions

15 Oxidation-based approaches are well known in the art. These include oxidation of p-alkoxybenzyl groups and the oxidation of sulfur and selenium linkers. The use of aqueous iodine to cleave disulfides and other sulfur or selenium-based linkers is also within the 20 scope of the invention.

F. Safety-catch linkers

Safety-catch linkers are those that cleave in two steps. In a preferred system the first step is the generation of a reactive nucleophilic center followed 25 by a second step involving an intra-molecular cyclization that results in cleavage. For example, levulinic ester linkages can be treated with hydrazine or photochemistry to release an active amine, which can then be cyclised to cleave an ester elsewhere in

the molecule (Burgess et al., J. Org. Chem. 62:5165-5168, 1997).

G. Cleavage by elimination mechanisms

5 Elimination reactions can also be used. For example, the base-catalysed elimination of groups such as Fmoc and cyanoethyl, and palladium-catalysed reductive elimination of allylic systems, can be used.

10 As well as the cleavage site, the linker can comprise a spacer unit. The spacer distances the nucleotide base from the cleavage site or label. The length of the linker is unimportant provided that the label is held a sufficient distance from the nucleotide so as not to interfere with any interaction between the nucleotide and an enzyme.

15 In a preferred embodiment the linker may consist of the same functionality as the block. This will make the deprotection and deblocking process more efficient, as only a single treatment will be required to remove both the label and the block.

20 A method for determining the sequence of a target polynucleotide can be carried out by contacting the target polynucleotide separately with the different nucleotides to form the complement to that of the target polynucleotide, and detecting the incorporation  
25 of the nucleotides. Such a method makes use of polymerisation, whereby a polymerase enzyme extends the complementary strand by incorporating the correct nucleotide complementary to that on the target. The polymerisation reaction also requires a specific  
30 primer to initiate polymerisation.

For each cycle, the incorporation of the modified nucleotide is carried out by the polymerase enzyme, and the incorporation event is then determined. Many different polymerase enzymes exist, and it will be  
5 evident to the person of ordinary skill which is most appropriate to use. Preferred enzymes include DNA polymerase I, the Klenow fragment, DNA polymerase III, T4 or T7 DNA polymerase, Taq polymerase or vent polymerase. A polymerase engineered to have specific  
10 properties can also be used.

The sequencing methods are preferably carried out with the target polynucleotide arrayed on a solid support. Multiple target polynucleotides can be immobilised on the solid support through linker  
15 molecules, or can be attached to particles, e.g., microspheres, which can also be attached to a solid support material.

The polynucleotides can be attached to the solid support by a number of means, including the use of  
20 biotin-avidin interactions. Methods for immobilizing polynucleotides on a solid support are well known in the art, and include lithographic techniques and "spotting" individual polynucleotides in defined positions on a solid support. Suitable solid supports  
25 are known in the art, and include glass slides and beads, ceramic and silicon surfaces and plastic materials. The support is usually a flat surface although microscopic beads (microspheres) can also be used and can in turn be attached to another solid  
30 support by known means. The microspheres can be of any suitable size, typically in the range of from 10 nm to 100 nm in diameter. In a preferred embodiment, the polynucleotides are attached directly



onto a planar surface, preferably a planar glass surface. Attachment will preferably be by means of a covalent linkage. Preferably, the arrays that are used are single molecule arrays that comprise  
5 polynucleotides in distinct optically resolvable areas, e.g., as disclosed in International Application No. WO00/06770.

The sequencing method can be carried out on both single polynucleotide molecule and multi-  
10 polynucleotide molecule arrays, i.e., arrays of distinct individual polynucleotide molecules and arrays of distinct regions comprising multiple copies of one individual polynucleotide molecule. Single molecule arrays allow each individual polynucleotide  
15 to be resolved separately. The use of single molecule arrays is preferred. Sequencing single molecule arrays non-destructively allows a spatially addressable array to be formed.

The method makes use of the polymerisation  
20 reaction to generate the complementary sequence of the target. The conditions necessary for polymerisation to occur will be apparent to the skilled person.

To carry out the polymerase reaction it will usually be necessary to first anneal a primer sequence  
25 to the target polynucleotide, the primer sequence being recognised by the polymerase enzyme and acting as an initiation site for the subsequent extension of the complementary strand. The primer sequence may be added as a separate component with respect to the  
30 target polynucleotide. Alternatively, the primer and the target polynucleotide may each be part of one single stranded molecule, with the primer portion

forming an intramolecular duplex with a part of the target, i.e., a hairpin loop structure. This structure may be immobilised to the solid support at any point on the molecule. Other conditions necessary  
5 for carrying out the polymerase reaction, including temperature, pH, buffer compositions etc., will be apparent to those skilled in the art.

The modified nucleotides of the invention are then brought into contact with the target  
10 polynucleotide, to allow polymerisation to occur. The nucleotides may be added sequentially, i.e., separate addition of each nucleotide type (A, T, G or C), or added together. If they are added together, it is preferable for each nucleotide type to be labelled  
15 with a different label.

This polymerisation step is allowed to proceed for a time sufficient to allow incorporation of a nucleotide.

Nucleotides that are not incorporated are then  
20 removed, for example, by subjecting the array to a washing step, and detection of the incorporated labels may then be carried out.

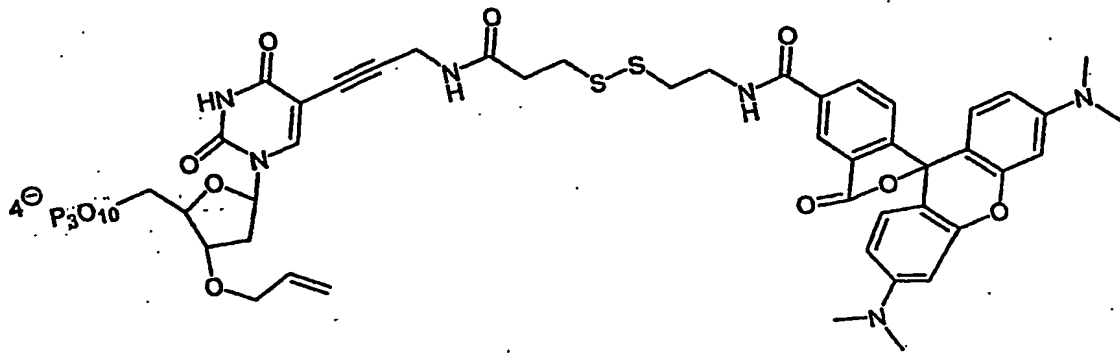
Detection may be by conventional means, for example if the label is a fluorescent moiety,  
25 detection of an incorporated base may be carried out by using a confocal scanning microscope to scan the surface of the array with a laser, to image a fluorophore bound directly to the incorporated base. Alternatively, a sensitive 2-D detector, such as a  
30 charge-coupled detector (CCD), can be used to visualise the individual signals generated. However, other techniques such as scanning near-field optical

microscopy (SNOM) are available and may be used when  
imaging dense arrays. For example, using SNOM,  
individual polynucleotides may be distinguished when  
separated by a distance of less than 100 nm, e.g., 10  
5 nm to 10  $\mu$ m. For a description of scanning near-field  
optical microscopy, see Moyer et al., *Laser Focus*  
*World* 29:10, 1993. Suitable apparatus used for  
imaging polynucleotide arrays are known and the  
technical set-up will be apparent to the skilled  
10 person.

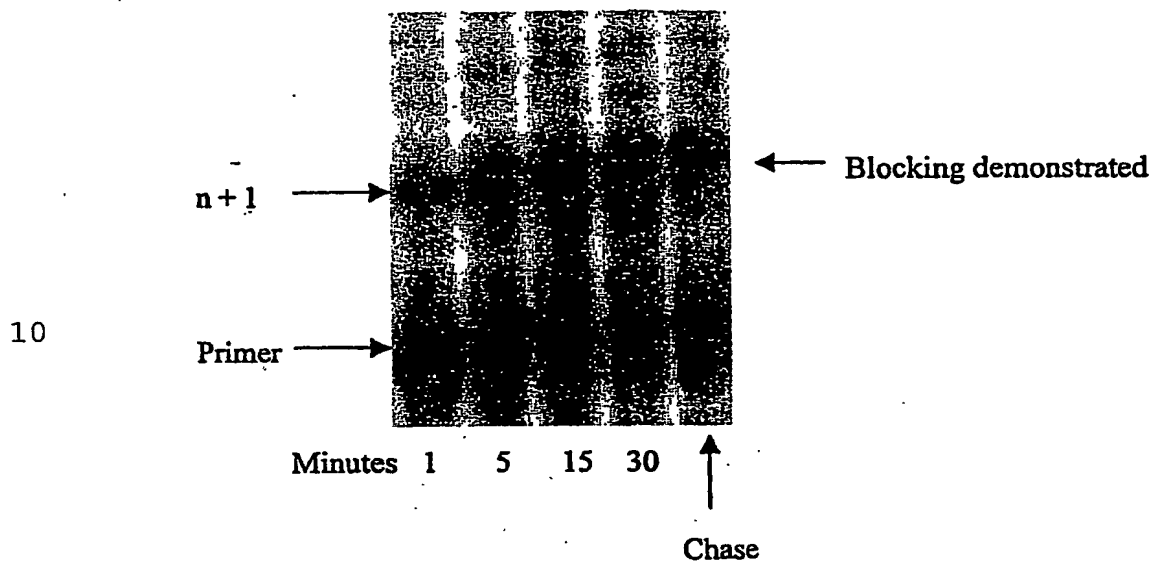
After detection, the label may be removed using  
suitable conditions that cleave the linker and the  
3'OH allyl block to allow for incorporation of further  
modified nucleotides of the invention. Appropriate  
15 conditions may be those described herein for allyl  
deprotection. These conditions serve to deprotect  
both the linker and the allyl group. Alternatively,  
the linker may be deprotected separately from the  
allyl group by employing methods of cleaving the  
20 linker known in the art (which do not sever the O-  
allyl bond) followed by deprotection.

#### EXAMPLE

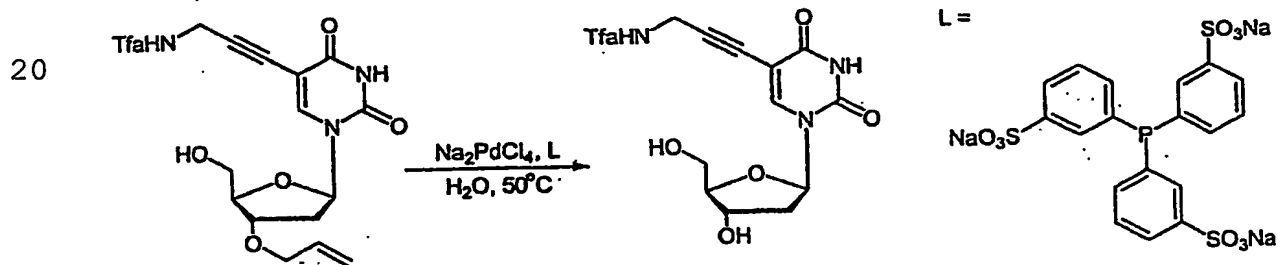
The following 3'O-allyl-protected nucleotide was used  
in enzyme incorporation and showed complete blocking  
25 against a 10A template:



Blocking against the action of *Thermococcus* sp. 9°N  
 exo -Y409V A485L was shown as is evident in the  
 following incorporation gel using at 10A template, in  
 which all four natural nucleotide were added in excess  
 5 in the chase experiment to verify complete blocking:



15 The following shows a typical deblocking procedure for  
 a 3'blocked nucleoside in which approximately 0.5  
 equivalents of  $\text{Na}_2\text{PdCl}_4$  and 4 equivalents of the  
 water-soluble phosphine ligand L were employed, in  
 water, at 50°C. Tfa stands for trifluoroacetyl:



25 The deprotection reaction was monitored by HPLC which  
 showed complete reaction after 30 minutes.

## Claims

1. A method of converting a compound of formula R-O-allyl,  $R_2N(allyl)$ ,  $RNH(allyl)$ ,  $RN(allyl)_2$  or R-S-allyl to a corresponding compound in which the allyl group is removed and replaced by hydrogen, said method comprising the steps of reacting a compound of formula R-O-allyl,  $R_2N(allyl)$ ,  $RNH(allyl)$ ,  $RN(allyl)_2$  or R-S-allyl in aqueous solution with a transition metal comprising a transition metal and one or more ligands selected from the group comprising water-soluble phosphine and water-soluble nitrogen-containing phosphine ligands, wherein the or each R is a water-soluble biological molecule.
2. The method of claim 1 wherein said compound is of formula R-O-allyl.
3. The method of claim 1 or claim 2 wherein said R is part of a nucleoside, a nucleotide or a polynucleotide.
4. The method of claim 3 wherein said nucleoside, a nucleotide or polynucleotide further comprises a detectable label linked to the base thereof by a cleavable linker.
5. The method of claim 4, wherein said detectable label is a fluorophore.
6. The method of claim 4 or 5 wherein said linker is acid labile, photolabile or contains a disulfide linkage.
7. The method of any one of claims 4 to 6 wherein said allyl group and said label are removed in a single step.

8. The method of any preceding claim wherein said transition metal is selected from the group comprising platinum, palladium, rhodium, ruthenium, osmium and iridium.

5 9. The method of any preceding claim wherein said transition metal is palladium.

10. The method of any preceding claim wherein said group of ligands comprise derivatised triaryl phosphine ligands.

10 11. The method of any preceding claim wherein said group of ligands are derivatised with one or more functionalities selected from the group comprising amino, hydroxyl, carboxyl and sulfonate groups.

15 12. The method of any preceding claim wherein the group of ligands comprises 3,3',3''-phosphinidynetris (benzenesulfonic acid).

13. The method of any preceding claim wherein said reacting step is in aqueous solution.

20 14. A nucleoside, nucleotide or polynucleotide of formula R-O-allyl, wherein R is said nucleoside or nucleotide or is a 3'terminal nucleotide of said polynucleotide; and said nucleoside or nucleotide further comprises a detectable label linked to the base thereof by a cleavable linker.

25 15. The nucleoside, nucleotide or polynucleotide of claim 14, wherein said detectable linker is a fluorophore.

16. The nucleoside, nucleotide or polynucleotide of claim 14 or claim 15, wherein said linker is acid labile, photolabile or contains a disulfide linkage.

5 17. A method of controlling the incorporation of a first nucleotide as defined in any one of claims 14 to 16 and complementary to a second nucleotide in a target single-stranded polynucleotide in a synthesis or sequencing reaction comprising incorporating into the growing complementary polynucleotide said first  
10 nucleotide, the incorporation of said first nucleotide preventing or blocking introduction of subsequent nucleoside or nucleotide molecules into said growing complementary polynucleotide.

15 18. The method of claim 17, wherein the incorporation of said first nucleotide is accomplished by a terminal transferase or polymerase or a reverse transcriptase.

19. A method for determining the sequence of a target single-stranded polynucleotide, comprising monitoring the sequential incorporation of complementary  
20 nucleotides, wherein at least one incorporation of the nucleotide is a nucleotide as defined in either of claims 15 or 16 and wherein the identity of the 3'-O-allyl nucleotide incorporated is determined by detecting the label linked to the base, and said  
25 blocking group and said label are removed prior to introduction of the next complementary nucleotide.

20. The method of claim 19 wherein the label of the nucleotide and the allyl group are removed in a single chemical treatment step.

30 21. A method for determining the sequence of a target single-stranded polynucleotide, comprising:

(a) providing a plurality of different 3'-O-allyl protected nucleotides as defined in either claim 15 or claim 16 and wherein the detectable label linked to each type of nucleotide can be distinguished upon detection from the detectable label used for other types of nucleotides;

(b) incorporating the nucleotide into the complement of the target single-stranded polynucleotide;

(c) detecting the label of the nucleotide of (b), thereby determining the type of nucleotide incorporated;

(d) removing the label of the nucleotide of (b) and the allyl blocking group; and

(e) optionally repeating steps (b)-(d) one or more times;

thereby determining the sequence of a target single-stranded polynucleotide.

22. The method of claim 21 wherein the label of the nucleotide and the allyl group are removed in a single chemical treatment step.

23. A method according to claim 21 or 22, wherein each of the nucleotides are brought into contact with the target sequentially, with removal of non-incorporated nucleotides prior to addition of the next nucleotide, and wherein detection and removal of the label and the allyl group is carried out either after addition of each nucleotide, or after addition of all four nucleotides.



24. A method according to claim 21 or 22, wherein each of the nucleotides are brought into contact with the target together simultaneously, and non-incorporated nucleotides are removed prior to  
5 detection and subsequent to removal of the label and the allyl group.

25. A method according to claim 21 or 22, comprising a first step and a second step, wherein in the first step, a first composition comprising two of the four  
10 nucleotides is brought into contact with the target and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label, and wherein in the second step, a second composition comprising the two nucleotides not included in the  
15 first composition is brought into contact with the target, and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label and allyl group, and wherein the first and second steps are optionally repeated one or more  
20 times.

26. A method according to claim 21 or 22 comprising a first step and a second step, wherein in the first step, a composition comprising one of the four  
25 nucleotides is brought into contact with the target, and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label and blocking group and wherein in the second step, a second composition comprising the three nucleotides not included in the first composition is brought into  
30 contact with the target, and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label and allyl group and

wherein the first steps and the second step are optionally repeated one or more times.

27. A method according to claim 25 comprising a first step and a second step, wherein in the first step, a  
5 first composition comprising three of the four nucleotides is brought into contact with the target, and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label and blocking group and wherein in the second step, a  
10 composition comprising the nucleotide not included in the first composition is brought into contact with the target, and non-incorporated nucleotides are removed prior to detection and subsequent to removal of the label and blocking group and wherein the first steps  
15 and the second step are optionally repeated one or more times.

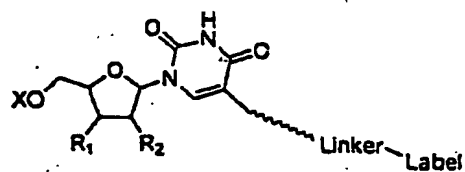
28. A kit, comprising:

(a) a plurality of different 3'O-allyl individual nucleotides as defined in either of claims  
20 15 or 16; and

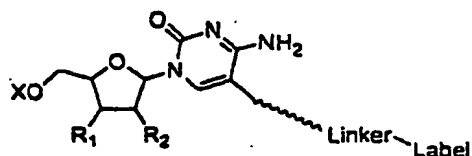
(b) packaging materials therefor.

29. A kit according to claim 28, wherein the detectable label in each nucleotide can be distinguished upon detection from the detectable label  
25 used for any of the other three types of nucleotide.

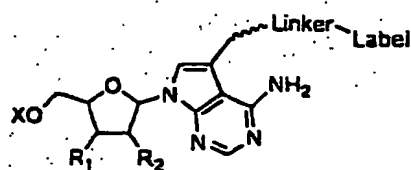
30. The kit of claim 28 or 29, further comprising an enzyme and buffers appropriate for the action of the enzyme.



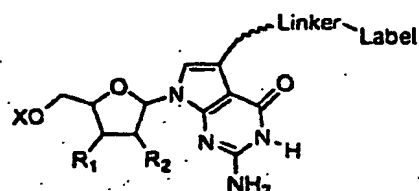
Uridine C5-linker



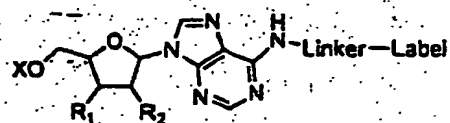
Cytidine C5-linker



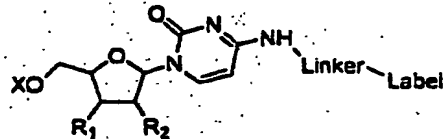
N7 Deazaadenosine C7-linker



N7 Deazaguanosine C7-linker



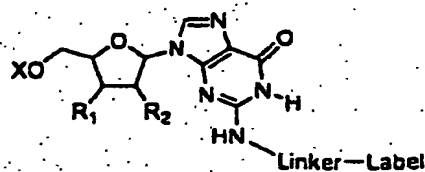
Adenosine N6-linker



Cytidine N4-linker

where  $R_1$  and  $R_2$ , which may be the same or different, are each selected from H, OH, or any group which can be transformed into an OH. Suitable groups for  $R_1$  and  $R_2$  are described in Figure 3

X = H, phosphate, diphosphate or triphosphate



Guanosine N2-linker

Fig. 1



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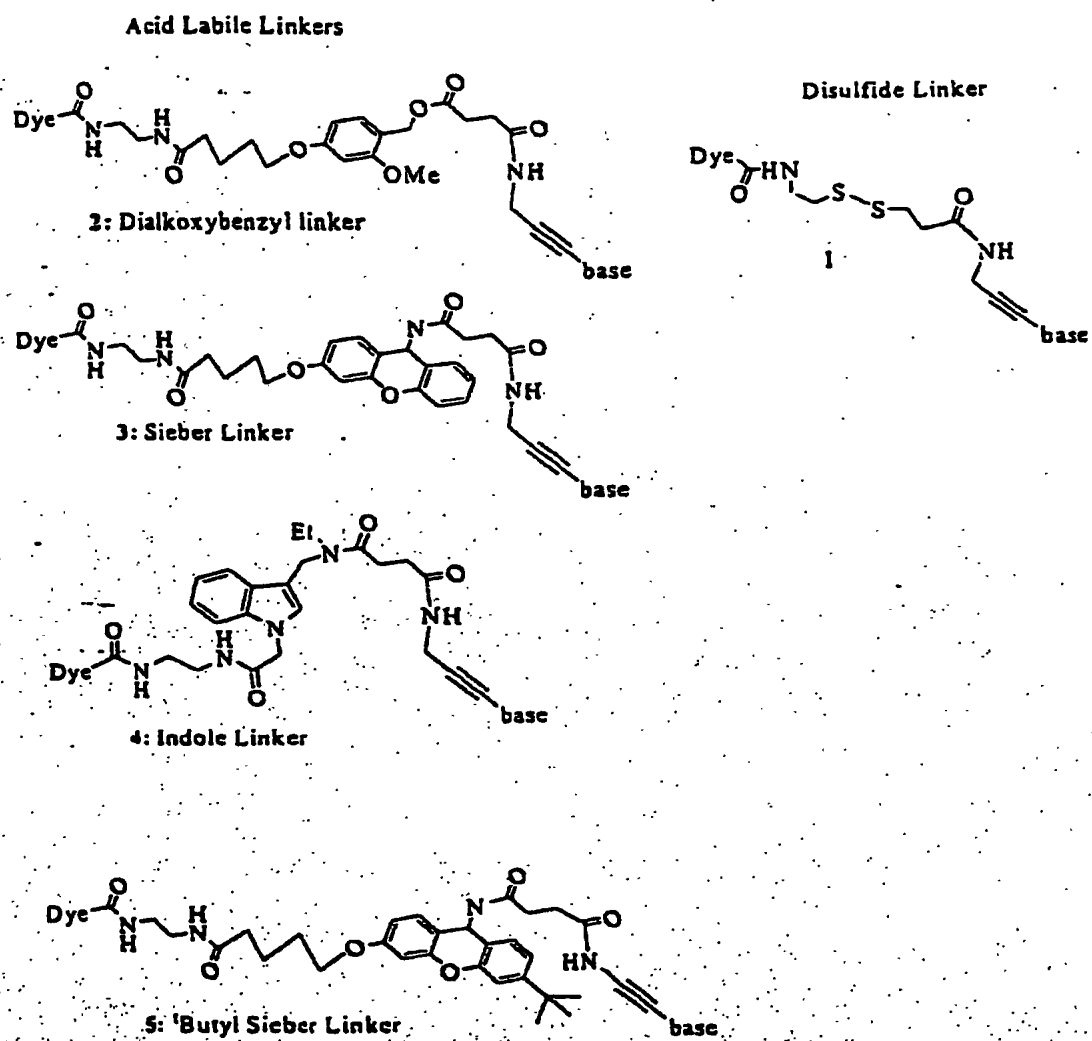
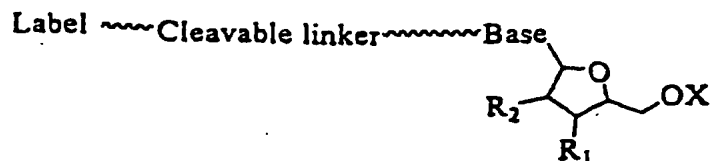
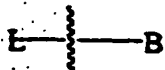


Fig. 2

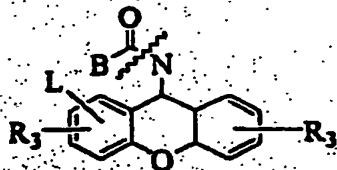
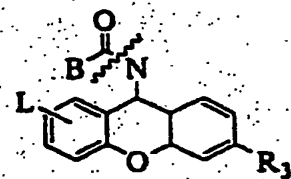
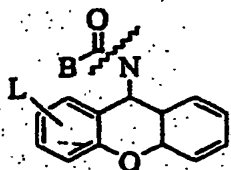
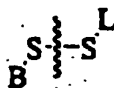
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where  $R_1$  and  $R_2$ , which may be the same or different, are each selected from H, OH, or any group which can be transformed into an OH, including a carbonyl



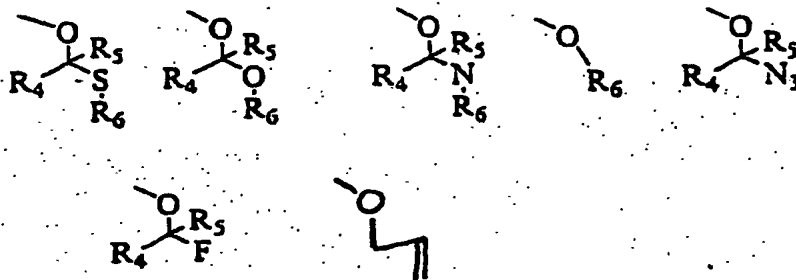
Cleavable linkers may include:



$R_3$  represents one or more substituents independently selected from alkyl, alkoxy, amino or halogen

Alternatively, cleavable linkers may be constructed from any labile functionality used on the 3'-block

$R_1$  and  $R_2$  groups may include



where  $R_4$  is H or alkyl;  $R_5$  is H or alkyl and  $R_6$  is alkyl, cycloalkyl, alkenyl, cycloalkenyl or benzyl

and X is H, phosphate, diphosphate or triphosphate

Fig. 3

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